



King's Research Portal

DOI:

[10.1111/odi.12866](https://doi.org/10.1111/odi.12866)

Document Version

Publisher's PDF, also known as Version of record

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

van Leewen, S.J.M., Proctor, G.B., Potting, C.M.J., ten Hoopen, S., van Groningen, L.F.J., Bronkhorst, E.M., Blijevens, N.M.A., & Huysmans, M.C.D.N.J.M. (2018). Early salivary changes in multiple myeloma patients undergoing autologous HSCT. *Oral Diseases*, 972-982. <https://doi.org/10.1111/odi.12866>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

ORIGINAL ARTICLE

Early salivary changes in multiple myeloma patients undergoing autologous HSCT

SJM van Leeuwen¹  | GB Proctor² | CMJ Potting³ | S ten Hoopen¹ |
LFJ van Groningen³ | EM Bronkhorst¹ | NMA Blijlevens³ | MCDNJM Huysmans¹¹Department of Dentistry, Radboudumc, Nijmegen, The Netherlands²Division of Mucosal & Salivary Biology, King's College London Dental Institute, London, UK³Department of Hematology, Radboudumc, Nijmegen, The Netherlands**Correspondence**Stephanie JM. van Leeuwen, Department of Dentistry, Radboudumc, Nijmegen, The Netherlands.
Email: Stephanie.vanLeeuwen@radboudumc.nl**Funding information**

Radboud Universitair Medisch Centrum

Objective: One explorative observational study in two parts was performed to examine early salivary changes in relation to oral mucositis (OM) in multiple myeloma patients treated with high-dose melphalan and autologous haematopoietic stem cell transplantation (HSCT). As cryotherapy was introduced after part A as regular care, its effect on OM could be evaluated.**Methods:** Unstimulated whole-mouth saliva (UWS) and stimulated whole-mouth saliva (SWS) were collected, and OM was scored with the Oral Mucositis Nursing Instrument (OMNI) at days -3, 0, 4, 7, 11 and 14 after HSCT. Salivary flow rate, total protein (BCA), mucin 5B, albumin (western blot), total IgA, lactoferrin and myeloperoxidase levels (ELISA) were determined.**Results:** Trends of decreasing UWS and SWS flow rates and total IgA levels were observed. At days 7 and 11, increases in lactoferrin and albumin levels were found in UWS and SWS. A positive correlation was found between OMNI scores and albumin and lactoferrin levels in SWS ($R^2 = .56$, $p = .029$ and $R^2 = .49$, $p = .043$, respectively). In part B, cryotherapy significantly lowered peak OMNI scores.**Conclusion:** Compositional changes in saliva reflecting inflammation were found in the first days after HSCT, and the use of cryotherapy in the second part was associated with decreased OM severity.**KEYWORDS**

haematopoietic stem cell transplantation, multiple myeloma, oral mucositis, saliva

1 | INTRODUCTION

Haematopoietic stem cell transplantation (HSCT) is a potentially curative treatment for malignant and non-malignant diseases. An HSCT can be performed either with autologous or allogeneic stem cells. In an autologous HSCT, patient's own stem cells are collected, stored and reinfused after suppressing the immune system with high-dose chemotherapy. For example, high-dose melphalan (HDM) is the standard care treatment regimen for multiple myeloma (MM) (Voorhees & Usmani, 2016).

A common adverse effect of HDM is oral mucositis (OM), characterized by pain, erythema, swollen mucosa, xerostomia and ulcerations (Peterson & Cariello, 2004). Severe OM requires narcotics and parenteral feeding and increases hospital stay. Furthermore, the ulcerative lesions may allow the penetration of bacteria into the bloodstream resulting in a systemic infection (Peterson & Cariello, 2004). Oral mucositis to some degree affects almost all patients treated with high-dose chemotherapy. A multicentre study to assess the incidence, severity and duration of OM found that 90% of the MM patients treated with

HDM and autologous HSCT was affected. Severe OM (WHO score 3 and 4) occurred in 46% of the MM patients and had a duration of 5 days (Blijlevens et al., 2008). Peak OM scores were found approximately 9 days after HSCT (Blijlevens et al., 2008). To prevent OM, cryotherapy is used for patients undergoing HDM and HSCT (Marchesi et al., 2017). Cryotherapy, the use of ice chips during the infusion of chemotherapy, results in local vasoconstriction, which reduces the amount of oncolytic drugs that reach the oral mucosa. Although it significantly reduces the incidence, some patients still develop severe OM (Marchesi et al., 2017). Moreover, there are no good treatment options for OM.

One factor that may influence the severity of OM is a decrease in the normal defence mechanisms in saliva (Epstein, Tsang, Warkentin, & Ship, 2002). Several salivary constituents are thought to have a protective function such as immunoglobulins, histatins, cystatins, defensins and lactoferrin (Amerongen & Veerman, 2002). A study evaluating changes in the salivary proteome after allogeneic HSCT found that levels of lactoferrin and secretory leucocyte protease inhibitor were increased at 6 months compared to pre-HSCT levels (Imanguli et al., 2007). The levels of secretory IgA (sIgA) were decreased 1 month after HSCT, but returned to pre-HSCT levels 6 months after HSCT (Imanguli et al., 2007). Decreased sIgA levels after HSCT were also found in another study examining salivary changes in MM patients undergoing autologous HSCT (Avivi et al., 2009). In the latter study, sIgA levels were significantly reduced 7 days after HSCT and OM was associated with a decrease in sIgA, uric acid levels and salivary antioxidant activity.

As OM is most severe around 9 days after transplantation, the aim of this study was to examine early salivary changes in relation to OM in MM patients treated with HDM and autologous HSCT. To determine salivary changes caused by leakage from the oral mucosae, unstimulated whole-mouth saliva (UWS) and stimulated (SWS) whole-mouth saliva were used in this explorative observational study performed in two parts. Due to protocol changes in regular care at the hospital based on the MASCC/ISOO clinical practice guideline in 2014 for the prevention of OM, patients in the last part received cryotherapy during infusion of HDM (Lalla et al., 2014).

2 | MATERIALS AND METHODS

An explorative observational study in 2 parts was performed in 2013 (part A) and 2015 (part B). Approval was given by the Local Ethical Committee (registration number 2012/528), which stated that this research was not subjected to the law governing research involving human subjects and no approval of the Local Ethical Committee was necessary. The study was conducted in full accordance of the World Medical Association (2001). All patients were informed about the study and signed informed consent before participating in the study.

2.1 | Study population

Adult MM patients undergoing HDM (200 mg/m², infused during 1 hr) and autologous HSCT at the Department of Hematology of the Radboud University Medical Centre were asked to participate. Patients who did not understand the Dutch language were excluded. In total, 12 (of 18) eligible patients were included from March 2013 to October 2013 for the first part (A) and 8 (of 11) eligible patients were included from May 2015 to July 2015 for the second part (B).

Patients brushed their teeth 4 times a day and rinsed with physiological salt solution when required per standard protocol. To maintain fluid balance during admission, patients received intravenous NaCl 0.9% solution. Analgesics, parenteral feeding and other medications were administered if necessary per standard protocol. In the second part, cryotherapy was used by consuming ice chips or crushed ice starting 5 min before HDM infusion (day -2) until 30 min after infusion. Melted ice was swallowed or spit out before the use of new ice chips. The medical charts were used to obtain the neutrophil counts and albumin levels in blood and to obtain the administered dose of melphalan in mg/kg.

2.2 | Oral examination and saliva collection

During the hospitalization period, saliva was collected and the severity of OM was scored using the OMNI score (Oral Mucositis Nursing Instrument) on day -3 (admission day), day 0 (day of autologous HSCT), day 4, day 7, day 11 and day 14 (Potting, Blijlevens, Donnelly, Feuth, & van Achterberg, 2006). The total OMNI score was calculated by the sum of the individual scores (ranging between 0 and 3) for erythema, edema, ulcerations, pain, mouth dryness and saliva viscosity.

The protocols for the collection of UWS and SWS were based on the guidelines for saliva collection of the University of Southern California School of Dentistry (Navazesh & Kumar, 2008). For the collection of UWS and SWS, patients were asked to refrain from eating or drinking 1 hr before collection and to swallow immediately before collection. For UWS, patients were asked to spit the saliva in a previously weighed plastic cup for 5 min without making any effort to increase the saliva flow. For SWS, patients were asked to chew 1 min on a piece of paraffin film to stimulate saliva flow. After swallowing, SWS collection was started by spitting saliva, while continuing chewing, in a plastic cup for 2-5 min. Directly after collecting, samples were weighed and stored on ice. UWS and SWS flow rates were estimated by assuming 1 ml of saliva is 1 gram. Directly after collection, pH was determined using pH strips (pH-indicator strips 4.0 - 7.0, Merck®, Germany), and saliva samples were centrifuged for 5 min at 9,800 g (mini spin plus, Eppendorf, Germany). The supernatant was stored separately from pellets at -80°C. For analysis, samples were defrosted and divided into aliquots and stored at -80°C.

2.3 | Saliva analysis

Total protein was determined using the BCA protein assay kit according to the manufacturer's protocol (Pierce™ BCA protein assay kit, Thermo Scientific, USA). Salivary proteins and glycoproteins were analysed by SDS-PAGE gels stained with Coomassie Brilliant Blue R250 (CBB) and Periodic Acid-Schiff (PAS) to determine mucin 5b (MUC5B). Western blotting was used to determine MUC5B and albumin levels, and enzyme-linked immunosorbent assay (ELISA) was used to determine total IgA, myeloperoxidase (MPO) and lactoferrin levels.

2.4 | SDS-PAGE and CBB/PAS staining

For SDS-PAGE gels, equal amounts of total protein were loaded onto gels, UWS and SWS separately. Samples were prepared in LDS containing sample buffer (1:4) and DTT (1:10), incubated at 95°C and centrifuged. Protein standards with predefined molecular weights (5 µl, SeeBlue® Plus2 prestained protein, Thermo Scientific, USA) and 10 µl of sample were loaded on 4%-12% Bis-Tris gels (NuPage™, Thermo Scientific, USA) and run in MES SDS running buffer as per manufacturer's recommendations. Gels were stained in CBB staining solution (Brilliant Blue R Staining Solution in 10% acetic acid, Sigma-Aldrich, UK) and destained in 10% acetic acid. The CBB-stained gels (after photographing) were used for PAS staining after 1 hr incubation in fixing solution (25% methanol, 10% glacial acetic acid in H₂O). After incubation, gels were washed with H₂O for 20 min, oxidized in 2% periodic acid for 15 min, washed with H₂O twice and incubated in Schiff reagent (VWR® Prolabo®, UK). Images were taken and band intensities were determined using a Bio-Rad® Chemidoc™ Imaging system and Image Lab™ software.

2.5 | Western blots

Using a sandwich technique, proteins were transferred from gel to a nitrocellulose membrane (Invitrogen, XCell II™ Blot Module, USA) in transfer buffer (Novex® NuPage Transfer Buffer 20x, USA, in methanol and H₂O) for 1 hr at constant voltage. After transfer, the membrane was blocked in 5% skimmed milk in TBS-T. Blots were incubated with primary mouse monoclonal anti-human MUC5B (1:500 in TBS-T, Abcam, Ab105460, UK) or mouse anti-albumin (1:1,000 in TBS-T Sigma, A6684, UK). Blots were washed 3x 5 min with TBS-T and incubated with second antibody, polyclonal goat anti-mouse HRP (1:2,000 in TBS-T, Dako P0447, UK). Results were visualized using a chemiluminescence Western blotting reagent (Bio-Rad® Clarity™ Western C Kit Western ECL Substrate, USA). Images were taken, and intensities were determined using Chemidoc and Image Lab™ software.

2.6 | Sandwich ELISA

Ninety-six well plates were coated overnight at 4°C (MPO at RT) with 100 µl rabbit anti-human IgA (1:1,000 in Na₂CO₃ buffer pH 9.6,

Dako A0262, UK), rabbit anti-human lactoferrin (1:2,000 in Na₂CO₃ buffer pH 9.6, Abcam Ab15811, UK) or MPO coating antibody (1:180 in PBS, Duoset DY3174, R&D systems, UK). The first antibody solution was discarded, and plates were washed 3x with PBS-T. Blocking with 300 µl of PBS with 1% BSA (1 hr at RT) was performed for MPO. After washing, 100 µl samples/standard was added in duplo to the plates and incubated at 37°C for 2 hr. IgA from colostrum I2636 (1 – 0.0156 µg/ml in PBS-T, Sigma-Aldrich, UK), lactoferrin from human milk L0520 (125 – 1.95 ng/ml in PBS-T, Sigma-Aldrich, UK) and MPO standard (16 – 0.25 ng/ml in PBS + 1% BSA, Duoset DY3174, R&D systems, UK) were used as standard. For total IgA and lactoferrin, samples were diluted 1:50 – 1:800 in PBS-T. For MPO, samples were diluted 1:20 – 1:400 in PBS + 1% BSA. After incubation, plates were washed 3x with PBS-T and incubated at 37°C for 1 hr (total IgA and lactoferrin) or at RT for 2 hr (MPO) with 100 µl of polyclonal anti-human IgA/HRP (1:4,000 in PBS-T, Dako 0216, UK), polyclonal rabbit anti-human lactoferrin/HRP (1:25000 in PBS-T, Abcam ab24264, UK) or detecting antibody (1:180 in PBS + 1% BSA, Duoset DY3174, UK). For MPO, plates were washed with PBS-T and incubated with 100 µl HRP solution (1:200 in PBS + 1% BSA, Duoset DY3174, UK) for 30 min at RT. After incubation with the second antibody or HRP solution, plates were washed with PBS-T and were incubated with 100 µl TMB (TMB liquid substrate system, Sigma-Aldrich, UK) for 30 min at RT in dark. Reaction was stopped with 50 µl 2 M sulphuric acid, and plates were read using a microplate reader at 492 nm (iMark™ Microplate Absorbance Reader Bio-Rad®, USA). Protein concentrations were calculated with Excel software using the standard curve.

2.7 | Data analysis

Statistical analysis was performed in SPSS (version 22) and R (version 3.4.0), and $p < .05$ was considered as statistical significant. Descriptive analysis was used for patient characteristics. To test the difference between the OMNI scores in part A and part B, a multi-level regression model was used with the OMNI score as dependent variable and "study part" as independent variable. To model the changes over time, both T and T^2 were included as independent variables as well, while a random intercept was added to the model to allow for the clustering of measurements within patients.

All quantitative results of MUC5B, albumin, total IgA, lactoferrin and MPO are based on part B only, as part A was used to optimize the protocols for quantitative analysis of saliva samples. Results from the salivary compositional analysis of the first part (A) were only used to compare trends between both studies. To correct for interindividual variation between patients, scaled scores were calculated per variable per patient by subtraction of day -3 score and dividing by the SD. These scaled scores were used for paired t tests to compare days 0, 4, 7, 11 and 14 with day -3. Differences are graphically labelled at α -levels of 5%, 1% and 0.1% separately to allow for evaluation of significance in view of multiple testing. For easier interpretation, most figures contain the absolute values. Correlations between peak OMNI scores and salivary parameters were calculated

by applying meta-analytical techniques (inverse variance weighting) on the individual correlations within patients over time.

3 | RESULTS

3.1 | Patient characteristics

In total, 20 patients were included (part A: $n = 12$, part B: $n = 8$). No important differences in study population were observed between both groups (Table 1). Due to hyposalivation or nausea, it was not possible to collect saliva from all patients on all study days. This resulted in missing samples with a peak at day 7 in both parts (Figure 1).

An important difference between both groups is the use of cryotherapy during the infusion of HDM in the second part (B). This resulted in significantly lower total OMNI scores in part B (95% CI [-3.87, -1.29], $p < .001$; Figure 2). Peak OMNI scores were seen at day 7 after HSCT, and the individual OMNI variables, mouth dryness and edema, contributed most to the total OMNI score each day (Figures 2 and 3). The highest individual OMNI scores for pain and lesions were found in part A (Figure 3).

TABLE 1 Patient characteristics

	First study ($n = 12$)	Second study ($n = 8$)
Gender (% male)	58.3%	62.5%
Age (median)	63	63
Range	43 – 68	53 – 67
Dentition (% dentate)	75%	62.5%
Melphalan doses in mg/kg (mean \pm SD)	4.96 \pm 0.60	4.65 \pm 0.86
Median	4.96	4.88

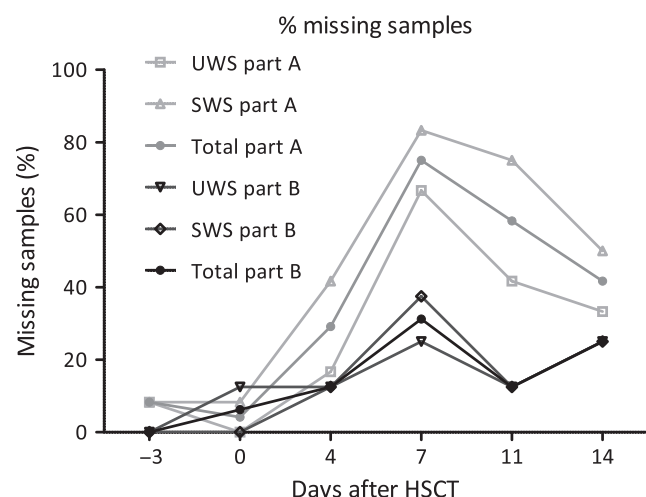


FIGURE 1 Percentage of missing samples over time for unstimulated whole-mouth saliva (UWS), stimulated whole-mouth saliva (SWS) and total (UWS + SWS) for part A (grey lines) and part B (black lines)

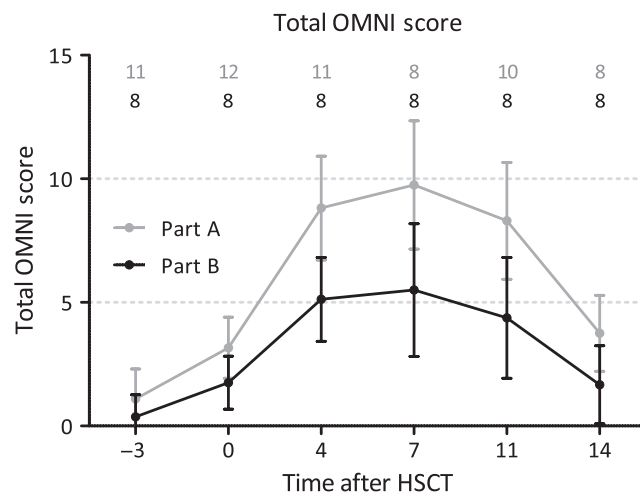


FIGURE 2 Total Oral Mucositis Nursing Instrument (OMNI) score. Mean \pm 95% CI of total OMNI score for part A (grey line) and part B (black line). Numbers in the graph represent the number of patients per time point for part A (grey) and part B (black). Total OMNI scores in part B were significantly lower compared to part A (95% CI [-3.87, -1.29], $p < .001$)

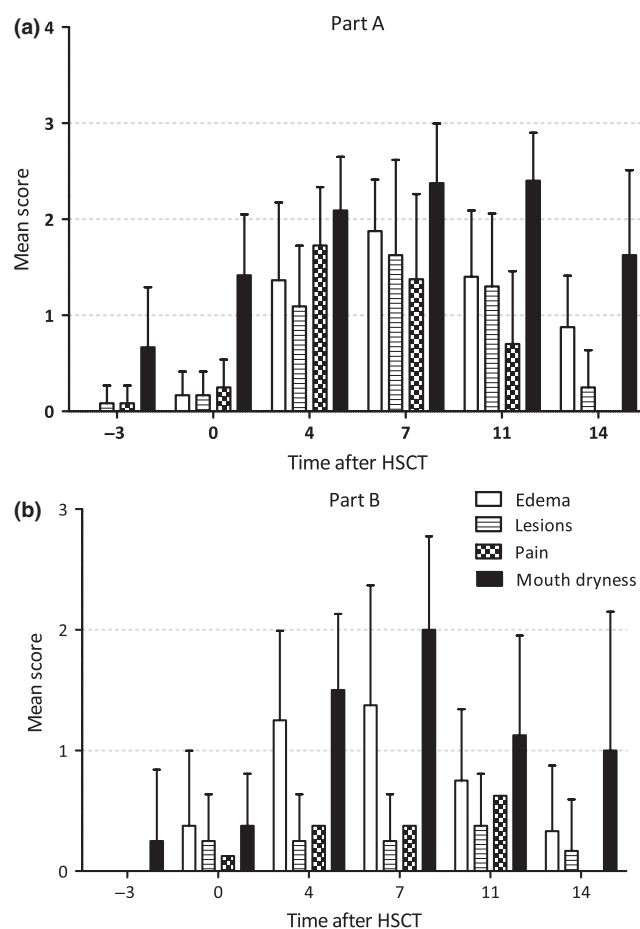


FIGURE 3 Contribution of four Oral Mucositis Nursing Instrument (OMNI) variables to total OMNI score per day. For each variable, mean score \pm 95% CI is shown for part A (a) and part B (b)

3.2 | Salivary flow rate, pH and total protein

Both parts showed similar changes in flow rate reductions for UWS and SWS over the study period, with generally flow rate levels being lower in part A (Figure 4a and b); especially for SWS, both parts consistently showed reduced flow rates at days 0, 4, 11 and 14, compared with day -3. The reductions were most clear in part B: SWS flow rate was significantly decreased at days 0, 4, 11 and 14 compared to day -3, 95% CI of scaled scores [-2.01, -0.84], $p < .001$; [-2.14, -0.77], $p = .002$; [-2.53, -1.96], $p < .001$; [-2.57, -1.37], $p < .001$, respectively. Salivary pH was determined directly after collection, showing a similar immediate reduction in UWS and SWS in part A and B at day 0 as seen in the flow rates: 95% CI of scaled scores [-2.11, -0.09], $p = .038$; [-2.51, -0.52], $p = .009$; [-2.57, -0.65], $p = .008$; [-2.18, -0.24], $p = .023$, for UWS and SWS part A and part B, respectively (Figure 5).

Although there is some indication of increasing total protein levels in both parts of the study, the effect is only consistent and statistically significant in SWS at day 0: 95% CI of scaled scores [0.92, 2.18], $p < .001$ for part A and [0.47, 2.37], $p = .011$ for part B (Figure 6).

3.3 | Salivary composition

3.3.1 | MUC5B + albumin

Figure 7a-c shows the changes in UWS and SWS MUC5B and albumin levels over time with a representative CBB- and PAS-stained SDS-PAGE and Western blot for MUC5B and albumin from one patient. Although in part A an increasing concentration of MUC5B was observed in UWS, this was not confirmed in part B (Figure 7b). Albumin levels showed a clear trend for both UWS and SWS, mimicking the curve of OMNI scores. The albumin levels were significantly increased at day 7 in

UWS and day 11 in SWS: 95% CI of scaled scores [0.57, 2.77], $p = .018$ and [0.61, 2.09], $p = .005$, respectively (Figure 7c). The albumin levels in blood showed a decreasing trend over time with lowest levels between days 9 and 10 after HSCT (Figure 7d).

3.3.2 | Total IgA, lactoferrin and MPO

Figure 8a-c shows the changes in UWS and SWS levels of total IgA, lactoferrin and MPO over time. A trend for decreasing and recovering concentration is seen in UWS and SWS total IgA levels; however, only at day 14 in UWS, the decrease was found statistically significant: 95% CI of scaled scores [-2.44, -0.23], $p = .032$ (Figure 8a). For lactoferrin, from day 4 an increasing trend was observed, which is almost parallel in UWS and SWS. The peak increase at day 11 is highly significant in both UWS and SWS: 95% CI of scaled scores [1.74, 2.56], $p < .001$ and [1.97, 2.58], $p < .001$, respectively. The preliminary part A showed similar trends for total IgA and lactoferrin. For MPO, a fluctuating trend is seen with only limited statistical significance of differences observed.

Myeloperoxidase was used as an indirect measurement for the polymorphonuclear leucocyte (PMN) levels in saliva. The PMN levels in blood are shown in Figure 8d. During the whole study period, a similar trend was observed between both parts. At day 0, an increase in PMN levels in blood was seen while the MPO levels in UWS were decreased. Directly after this increase in blood PMNs, a decreasing trend is seen reaching undetectable levels at day 6 after HSCT as expected (Figure 8c and d). At day 4, MPO levels in UWS showed an increase compared to day 0, while the PMN levels in blood were decreased compared to day 0 (Figure 8c and d). At day 7, no PMNs were detected in blood and the lowest MPO levels in UWS were found. Interestingly, the UWS MPO levels started to increase at day 11, while the PMNs were still undetectable in blood (Figure 8c and d).

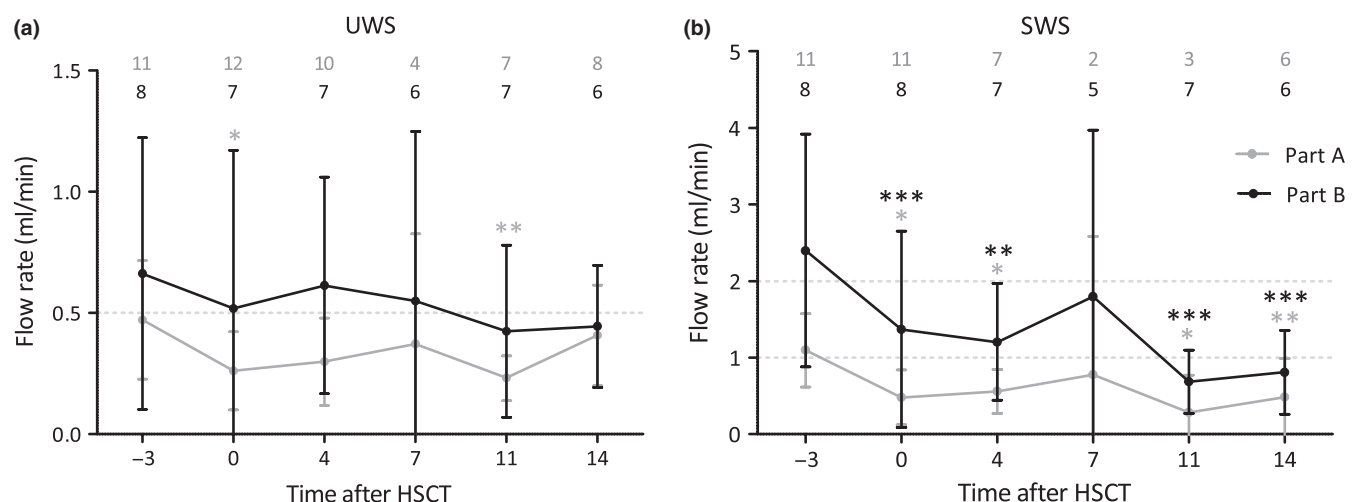


FIGURE 4 Mean \pm 95% CI of unstimulated whole-mouth saliva (UWS) (a) and stimulated whole-mouth saliva (SWS) (b) flow rate over time for part A (grey lines) and part B (black lines). Numbers in the graphs represent the number of patients per time point for part A (grey) and part B (black). Significance levels are based on paired t tests with scaled scores all compared to day -3, * $p < .05$, ** $p < .01$ and *** $p < .001$

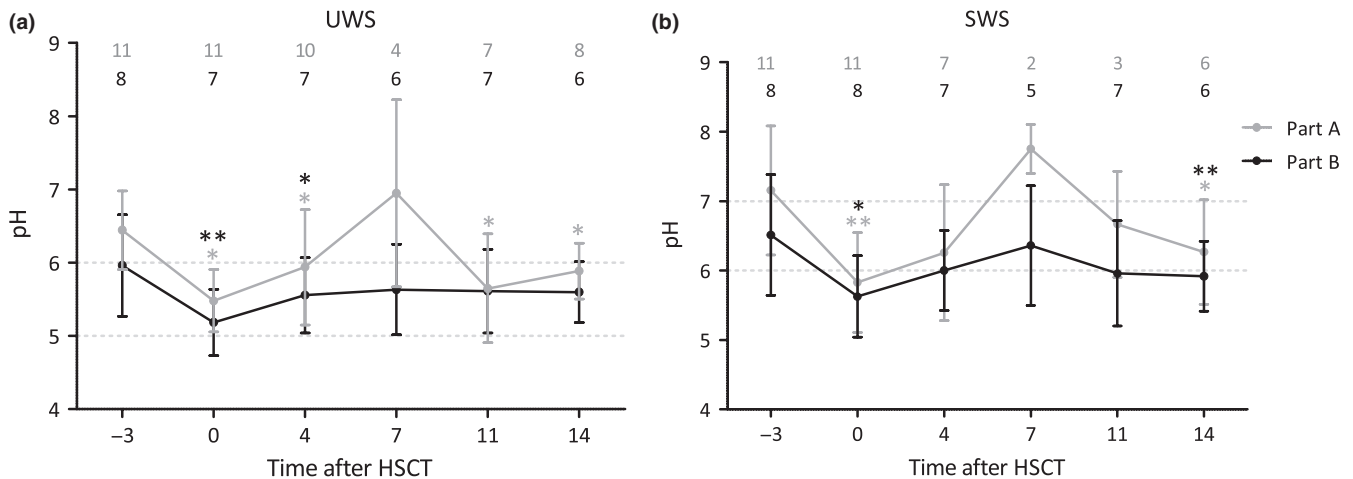


FIGURE 5 Mean \pm 95% CI for unstimulated whole-mouth saliva (UWS) (a) and stimulated whole-mouth saliva (SWS) (b) pH of part A (grey lines) and part B (black lines). Numbers in the graphs represent the number of patients per time point for part A (grey) and part B (black). Significance levels are based on paired t tests with scaled scores all compared to day -3, * $p < .05$, ** $p < .01$

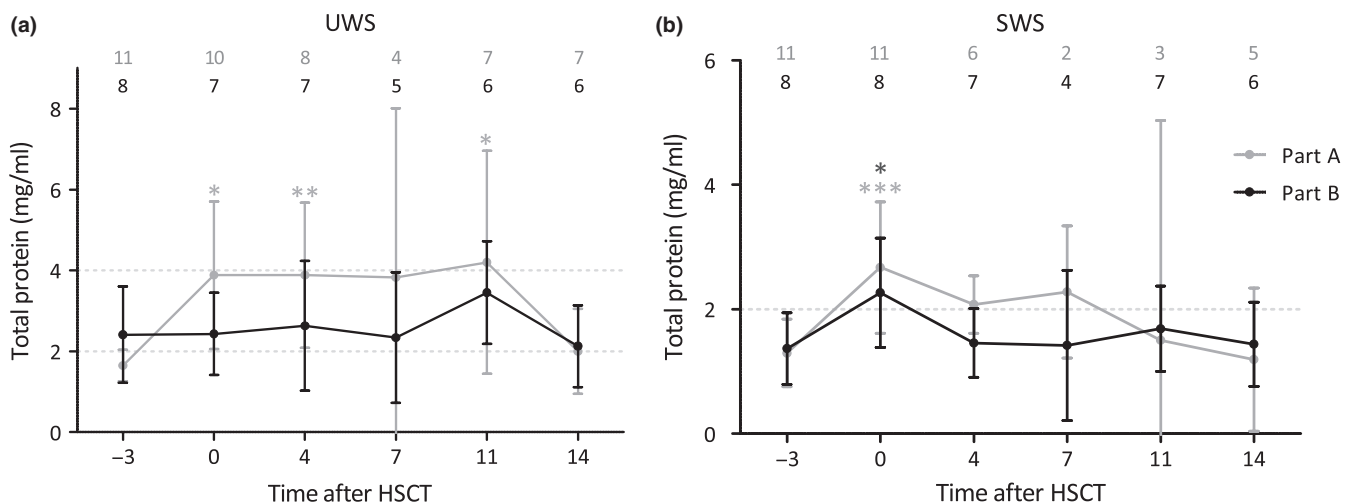


FIGURE 6 Mean \pm 95% CI of total protein concentration in unstimulated whole-mouth saliva (UWS) (a) and stimulated whole-mouth saliva (SWS) (b) for part A (grey lines) and part B (black lines). Numbers in the graphs represent the number of patients per time point for part A (grey) and part B (black). Significance levels are based on paired t tests with scaled scores all compared to day -3, * $p < .05$, ** $p < .01$, *** $p < .001$

3.4 | Correlations

Correlations between OMNI scores and salivary parameters are shown in Table 2. A positive correlation was found between total OMNI scores and SWS MUC5B, SWS albumin and SWS lactoferrin levels ($R^2 = .318$, $p = .043$; $R^2 = .56$, $p = .029$ and $R^2 = .49$, $p = .043$, respectively). In addition, total OMNI scores and UWS albumin levels were close to a statistical significant positive correlation ($R^2 = .47$, $p = .05$), and total OMNI scores and SWS flow rate were close to a statistical significant negative correlation ($R^2 = -.39$, $p = .089$).

As oral dryness is the largest contributor to OMNI scores in this study (Figure 2b), correlations were also determined between the dryness component of the OMNI score and flow rate and MUC5B levels in UWS and SWS (Table 2). A negative correlation was found between UWS flow rate and the dryness component of the OMNI score ($R^2 = -.57$, $p = .003$), and a positive correlation

was found between SWS MUC5B levels and the dryness component of the OMNI score ($R^2 = .51$, $p = .015$), indicating that the increased feeling of a dry mouth starting from day 0 was correlated with the trend of increasing SWS MUC5B levels between days 0 and 7.

4 | DISCUSSION

This study was performed to determine early changes in salivary parameters in relation to OM in MM patients undergoing autologous HSCT. After HSCT, salivary albumin and lactoferrin levels were increased, whereas salivary flow rate showed a decreasing trend. A positive correlation was found between OM and MUC5B, albumin and lactoferrin levels in SWS. Furthermore, due to protocol changes it was possible to observe the effect of cryotherapy on OM

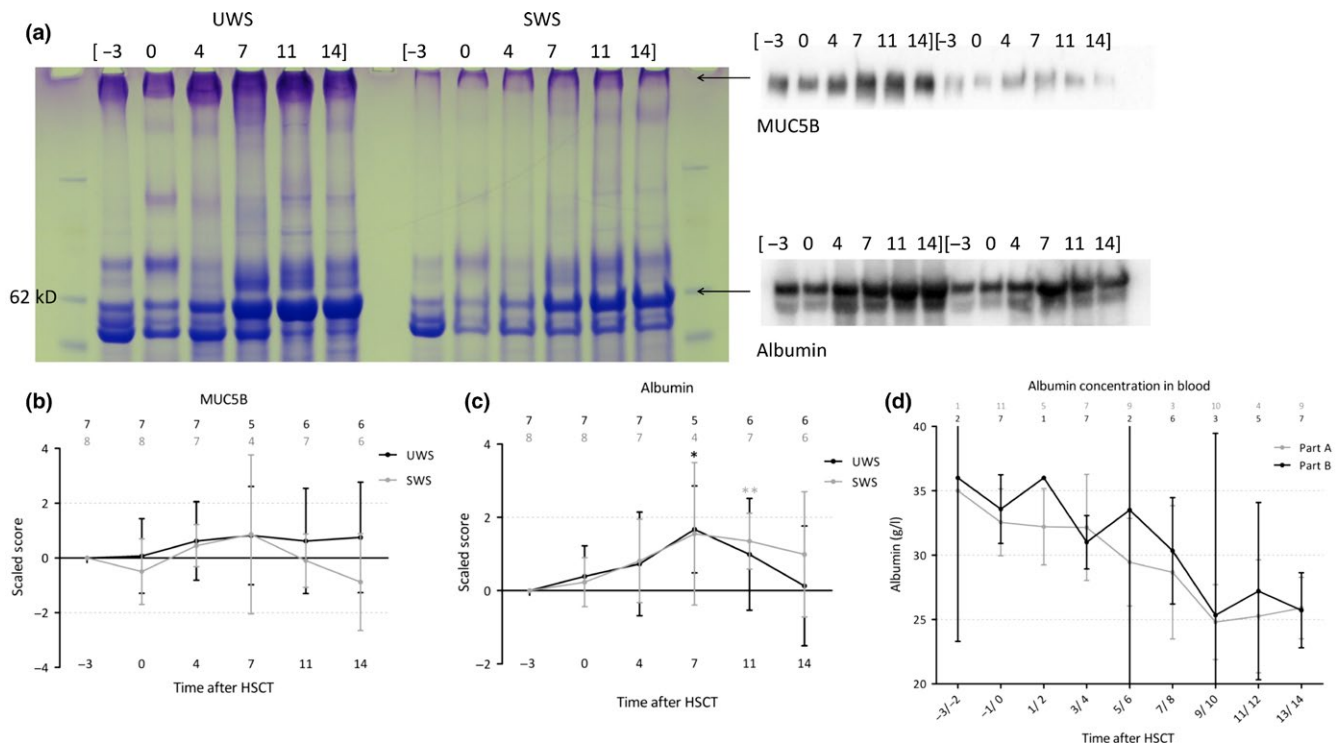


FIGURE 7 Differences in unstimulated whole-mouth saliva (UWS) and stimulated whole-mouth saliva (SWS) MUC5B and albumin. (a) Example of a Coomassie Brilliant Blue (CBB)- and Periodic Acid-Schiff (PAS)-stained gel and Western blots for MUC5B and albumin of one representative patient. Mean \pm 95% CI of scaled scores of MUC5B (b) and albumin (c) levels in UWS (black lines) and SWS (grey lines). Numbers in the graphs represent the number of patients per time point for UWS (black) and SWS (grey). Significance levels are based on paired t tests with scaled scores compared to day -3. * $p < .05$, ** $p < .01$. (d) Mean \pm SD of albumin concentration in blood for part A (grey line) and part B (black line), numbers in the graph represent the number of patients per time point for part A (grey) and part B (black) [Colour figure can be viewed at wileyonlinelibrary.com]

development, which resulted in significantly lower max OMNI scores during the whole study period in the second part with cryotherapy.

Consistent with the published literature, peak OM was reached around day 7 and had almost returned to baseline at the end of the study period (Blijlevens et al., 2008). Oral mucositis caused by HDM was reduced by the use of cryotherapy in this study. As melphalan is found in saliva, the local vasoconstriction caused by the cryotherapy may reduce the melphalan concentration in saliva (Epstein et al., 2002; Slavik, Wu, & Riley, 1993). Similar to a retrospective study, cryotherapy resulted in reduced OM, but did not reduce the duration of OM (Batlle et al., 2014).

Although a direct effect of HDM on salivary glands is unclear, conditioning therapy before HSCT causes salivary hypofunction (Epstein et al., 2002). Furthermore, salivary gland dysfunction by lymphocyte infiltration is found using gland biopsies and a scintigraphic method several weeks after HSCT with conditioning regimens consisting of cyclophosphamide, busulfan and TBI (Coracin et al., 2006; Lindahl, Lönnquist, & Hedfors, 1988). Most other studies measuring salivary flow rate after HSCT found a decrease in salivary flow rate (Amaral et al., 2012; Avivi et al., 2009; Boer, Correa, Tenuta, Souza, & Vigorito, 2015; Elad et al., 2006; Nagler, Barness-Hadar, Lieba, & Nagler, 2006). However, one study found no changes in UWS flow rate in autologous HSCT patients, while a study in allogeneic patients showed an increase in UWS flow rate

8-10 days after HSCT (Avivi et al., 2009; Boer et al., 2015). This increase might be related to dysphagia resulting in an increased resting volume of saliva as suggested by the authors (Boer et al., 2015).

One indication for glandular dysfunction caused by HDM is the similar decrease in flow rate and pH at day 4 in this study. However, the protein secretory functions of the glands are not affected as suggested by the increase in total protein and lactoferrin. Lactoferrin in saliva has a glandular origin although it is also a product of PMNs (Reitamo, Kontinen, & Segerberg-Kontinen, 1980). The increase in lactoferrin observed in this study may be derived from glandular origin as the PMN levels in blood decrease after HSCT resulting in neutropenia. To further investigate protein output of the salivary glands, salivary amylase levels could be studied or glandular saliva could be used to determine changes in specific glandular-derived proteins.

Myeloperoxidase is secreted by PMNs and could therefore be used as an indirect measurement of the PMN levels in saliva. The decrease in MPO found in this study at day 7 probably reflects neutropenia. Interestingly, the MPO levels in saliva showed an increasing trend some days before the PMNs were again detectable in blood. This finding is supported by two other studies measuring PMN levels directly after HSCT and may indicate that PMNs from the stem cells directly reach the site of inflammation before they circulate at detectable levels in blood (Lieschke et al., 1992; Pink et al., 2009).

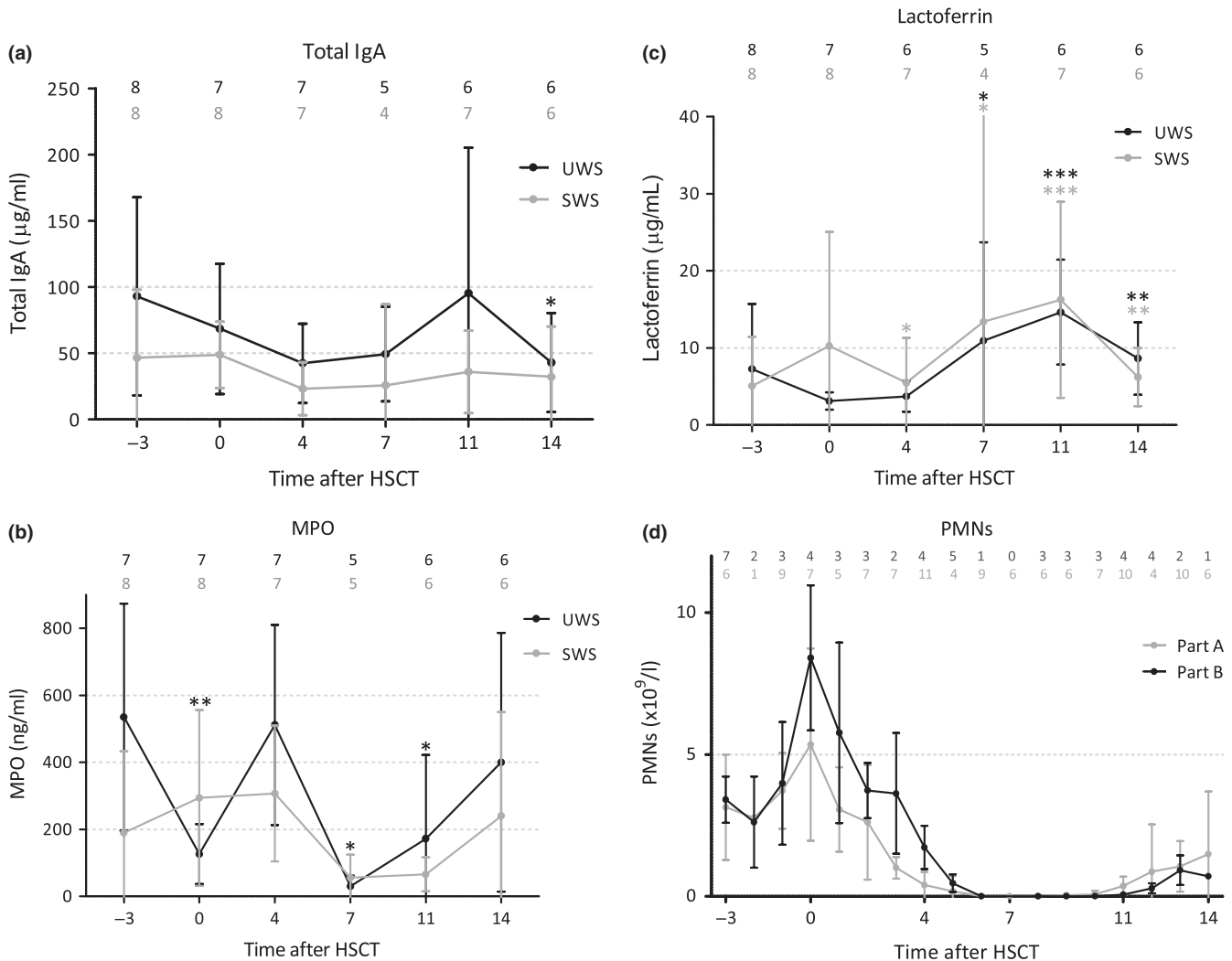


FIGURE 8 Mean \pm 95% CI of absolute values of total IgA (a), lactoferrin (b) and myeloperoxidase (c) levels in unstimulated whole-mouth saliva (UWS) (black lines) and stimulated whole-mouth saliva (SWS) (grey lines). Numbers in the graphs represent the number of patients per time point for UWS (black) and SWS (grey). Significance levels are based on paired t tests with scaled scores all compared to day -3, * $p < .05$, ** $p < .01$ and *** $p < .001$. (d) Mean \pm SD of blood levels of polymorphonuclear leucocytes (PMNs) over time for part A (grey line) and part B (black line), numbers in the graph represent the number of patients per time point for part A (grey) and part B (black)

Besides a decreasing trend in salivary flow rate, a decrease in salivary antimicrobial defence is also described in the literature mainly by a decrease in total or secretory IgA levels after HSCT (Avivi et al., 2009; Chaushu et al., 1994; Dens et al., 1996; Norhagen-Engstrom, Hammarstrom, Lonnqvist, Soder, & Smith, 1988). One mechanism to overcome this decreased antimicrobial defence might be an increased secretion of lactoferrin as most salivary proteins act in synergy for the antimicrobial function (Avivi et al., 2009; Imanjuli et al., 2007). This hypothesis is supported in our study by a decreasing trend in total IgA levels and increased levels of lactoferrin. Furthermore, the increase in lactoferrin and albumin levels was also positively correlated with OM. Salivary albumin levels were increased during peak OM, while blood levels of albumin show a decreasing trend. This supports the previously described hypothesis of decreased integrity of the oral mucosa, which may result in leakage of blood substances into saliva during OM (Avivi et al., 2009; Izutsu et al., 1981).

Another OM-related complaint after HSCT is xerostomia, the feeling of a dry mouth. As suggested by the dryness component of the OMNI score, xerostomia was present during the whole study period. Xerostomia has been linked to changes in the lubricating protein MUC5B (Chaudhury, Shirlaw, Pramanik, Carpenter, & Proctor, 2015; Dijkema et al., 2012). The dryness component of the OMNI score was negatively correlated with UWS flow rate and, although no significant changes in MUC5B concentrations were observed, positively correlated with SWS MUC5B levels. This counterintuitive finding is supported by another study where patients with xerostomia had similar or higher MUC5B and MUC7 concentrations, combined with differences in the glycosylation of the mucins (Chaudhury et al., 2015). We conclude that the correlation may be based on reduced total MUC5B availability or changed lubricating capabilities of the mucins.

The differences in the levels of proteins between UWS and SWS found in this study can be explained by the normal composition of

TABLE 2 Correlation between Oral Mucositis Nursing Instrument (OMNI) scores and salivary parameters, based on inverse variance weighting of correlations within patients over time from part B

Variable	Heterogeneity I^2 (%); p	Correlation coefficient	95% CI	Significance (two-tailed)
Total OMNI - UWS				
Flow rate	0; 0.96	-0.33	-0.79, 0.14	.138
MUC5B	0; 0.78	0.14	-0.33, 0.62	.483
Albumin	0; 0.92	0.47	0, 0.95	.050
IgA	0; 0.84	0.07	-0.41, 0.54	.749
Lactoferrin	0; 0.97	0.24	-0.23, 0.72	.254
MPO	0; 0.94	-0.21	-0.69, 0.26	.310
Total OMNI - SWS				
Flow rate	0; 0.92	-0.39	-0.87, 0.08	.089
MUC5B	0; 0.99	0.49	0.02, 0.97	.043*
Albumin	0; 0.98	0.56	0.08, 1.03	.029*
IgA	0; 0.85	0.2	-0.28, 0.67	.354
Lactoferrin	0; 0.97	0.49	0.02, 0.97	.044*
MPO	0; 0.88	0.04	-0.43, 0.52	.824
OMNI dryness - UWS				
Flow rate	0; 0.59	-0.57	-0.87, -0.28	.003**
MUC5B	0; 0.65	0.3	-0.05, 0.65	.081
OMNI dryness - SWS				
Flow rate	49; 0.07	-0.28	-0.73, 0.16	.168
MUC5B	36; 0.15	0.51	0.14, 0.87	.015*

MPO, myeloperoxidase; SWS, stimulated whole-mouth saliva; UWS, unstimulated whole-mouth saliva.

* $p < .05$.

** $p < .01$.

UWS and SWS. In resting conditions, saliva is mainly formed by secretions from the submandibular and sublingual glands. In stimulated saliva, the parotid gland contributes more, which results in more watery saliva compared to UWS (Proctor, 2016).

One limitation of several studies evaluating salivary changes in HSCT recipients is the mixed patient population. Only one earlier study evaluated early salivary changes in a homogenous group of MM patients receiving HDM and autologous HSCT (Avivi et al., 2009). Our observational study also included a homogenous patient population. However, both studies included small numbers of patients and lost power due to missing samples caused by nausea and hyposalivation especially during the OM period. Furthermore, due to logistic difficulties it was not possible to collect saliva at the same day during the whole study period. It was tried to collect saliva at the same time per patients, but also due to logistic difficulties, this was not always possible.

Salivary changes may influence the severity of OM. For proper symptom management, local salivary changes may be indicative. This study found decreased total IgA levels in saliva resulting in decreased antimicrobial defence, which may be compensated by other antimicrobial proteins in saliva such as lactoferrin. However, further research is necessary to confirm and expand these results.

In conclusion, our observational study showed compositional changes in saliva reflecting inflammation of the salivary glands and

the oral mucosae in the first days after autologous HSCT, of which the changes in albumin and lactoferrin were correlated with OM development. Although cryotherapy in the second study resulted in decreased OM severity, both studies showed similar trends in altered salivary protein composition.

ACKNOWLEDGEMENTS

The study was funded by the Radboudumc in Nijmegen (The Netherlands). For the optimization of the laboratory protocols to determine protein concentrations, we want to thank G.W. Hameete.

CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTION

The study was designed by C.M.J. Potting, N.M.A. Blijlevens, M.C.D.N.J.M. Huysmans and L.F.J. van Groningen. S. ten Hoopen had a substantial contribution in the data collection for the clinical and the laboratory part. The laboratory part was performed by S. ten Hoopen at the lab of G.B. Proctor. For the optimization of the laboratory protocols G.B. Proctor had a substantial contribution.

The data analysis was performed by S.J.M. van Leeuwen, G.B. Proctor, S. ten Hoopen and E.M. Bronkhorst. S.J.M. van Leeuwen drafted the paper, which was reviewed by G.B. Proctor, C.M.J. Potting, N.M.A. Blijlevens, M.C.D.N.J.M. Huysmans and L.F.J. van Groningen.

ORCID

SJM Leeuwen  <http://orcid.org/0000-0002-4522-3705>

REFERENCES

- Amaral, T. M. P., Campos, C. C., Dos Santos, T. P. M., Leles, C. R., Teixeira, A. L., Teixeira, M. M., ... Da Silva, T. A. (2012). Effect of salivary stimulation therapies on salivary flow and chemotherapy-induced mucositis: A preliminary study. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 113, 628–637. <https://doi.org/10.1016/j.oooo.2011.10.012>
- Amerongen, A. V., & Veerman, E. C. (2002). Saliva—the defender of the oral cavity. *Oral Diseases*, 8, 12–22. <https://doi.org/10.1034/j.1601-0825.2002.1o816.x>
- Avivi, I., Avraham, S., Koren-Michowitz, M., Zuckerman, T., Aviv, A., Ofra, Y., ... Nagler, R. M. (2009). Oral integrity and salivary profile in myeloma patients undergoing high-dose therapy followed by autologous SCT. *Bone Marrow Transplantation*, 43, 801–806. <https://doi.org/10.1038/bmt.2008.387>
- Batlle, M., Morgades, M., Vives, S., Ferra, C., Oriol, A., Sancho, J. M., ... Ribera, J. M. (2014). Usefulness and safety of oral cryotherapy in the prevention of oral mucositis after conditioning regimens with high-dose melphalan for autologous stem cell transplantation for lymphoma and myeloma. *European Journal of Haematology*, 93, 487–491. <https://doi.org/10.1111/ejh.12386>
- Blijlevens, N., Schwenkglenks, M., Bacon, P., D'Addio, A., Einsele, H., Maertens, J., ... European Blood and Marrow Transplantation Mucositis Advisory Group. (2008). Prospective oral mucositis audit: Oral mucositis in patients receiving high-dose melphalan or BEAM conditioning chemotherapy—European Blood and Marrow Transplantation Mucositis Advisory Group. *Journal of Clinical Oncology*, 26, 1519–25. <https://doi.org/10.1200/JCO.2007.13.6028>
- Boer, C. C., Correa, M. E., Tenuta, L. M., Souza, C. A., & Vigorito, A. C. (2015). Post-allogeneic Hematopoietic Stem Cell Transplantation (HSCT) changes in inorganic salivary components. *Supportive Care in Cancer*, 23, 2561–2567. <https://doi.org/10.1007/s00520-015-2613-0>
- Chaudhury, N., Shirlaw, P., Pramanik, R., Carpenter, G., & Proctor, G. (2015). Changes in saliva rheological properties and mucin glycosylation in dry mouth. *Journal of Dental Research*, 94, 1660–1667. <https://doi.org/10.1177/0022034515609070>
- Chaushu, S., Chaushu, G., Garfunkel, A. A., Slavin, S., Or, R., & Yefenof, E. (1994). Salivary immunoglobulins in recipients of bone marrow grafts. I A longitudinal follow-up. *Bone Marrow Transplantation*, 14, 871–876.
- Coracin, F. L., Pizzigatti Correa, M. E., Camargo, E. E., Peterson, D. E., de Oliveira Santos, A., Vigorito, A. C., ... De Souza, C. A. (2006). Major salivary gland damage in allogeneic hematopoietic progenitor cell transplantation assessed by scintigraphic methods. *Bone Marrow Transplantation*, 37, 955–959. <https://doi.org/10.1038/sj.bmt.1705351>
- Dens, F., Boogaerts, M., Boute, P., Declerck, D., Demuyne, H., & Vincikier, F. (1996). Quantitative determination of immunological components of salivary gland secretion in transplant recipients. *Bone Marrow Transplantation*, 17, 421–423.
- Dijkema, T., Terhaard, C. H., Roesink, J. M., Raaijmakers, C. P., van den Keijbus, P. A., Brand, H. S., & Veerman, E. C. (2012). MUC5B levels in submandibular gland saliva of patients treated with radiotherapy for head-and-neck cancer: A pilot study. *Radiation Oncology*, 7, 91. <https://doi.org/10.1186/1748-717X-7-91>
- Elad, S., Ackerstein, A., Bitan, M., Shapira, M. Y., Resnick, I., Gesundheit, B., ... Or, R. (2006). A prospective, double-blind phase II study evaluating the safety and efficacy of a topical histamine gel for the prophylaxis of oral mucositis in patients post hematopoietic stem cell transplantation. *Bone Marrow Transplantation*, 37, 757–762. <https://doi.org/10.1038/sj.bmt.1705331>
- Epstein, J. B., Tsang, A. H., Warkentin, D., & Ship, J. A. (2002). The role of salivary function in modulating chemotherapy-induced oropharyngeal mucositis: A review of the literature. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, 94, 39–44. <https://doi.org/10.1067/moe.2002.126018>
- Imanguli, M. M., Atkinson, J. C., Harvey, K. E., Hoehn, G. T., Ryu, O. H., Wu, T., ... Hart, T. C. (2007). Changes in salivary proteome following allogeneic hematopoietic stem cell transplantation. *Experimental Hematology*, 35, 184–192. <https://doi.org/10.1016/j.exphem.2006.10.009>
- Izutsu, K. T., Truelove, E. L., Bleyer, W. A., Anderson, W. M., Schubert, M. M., & Rice, J. C. (1981). Whole saliva albumin as an indicator of stomatitis in cancer therapy patients. *Cancer*, 48, 1450–1454. [https://doi.org/10.1002/\(ISSN\)1097-0142](https://doi.org/10.1002/(ISSN)1097-0142)
- Lalla, R. V., Bowen, J., Barasch, A., Elting, L., Epstein, J., Keefe, D. M., ... Peterson, D. E. (2014). MASCC/ISOO clinical practice guidelines for the management of mucositis secondary to cancer therapy. *Cancer*, 120, 1453–1461. <https://doi.org/10.1002/cncr.28592>
- Lieschke, G. J., Ramenghi, U., O'Connor, M. P., Sheridan, W., Szer, J., & Morstyn, G. (1992). Studies of oral neutrophil levels in patients receiving G-CSF after autologous marrow transplantation. *British Journal of Haematology*, 82, 589–595. <https://doi.org/10.1111/j.1365-2141.1992.tb06472.x>
- Lindahl, G., Lönnquist, B., & Hedfors, E. (1988). Lymphocytic infiltration and HLA-DR expression of salivary glands in bone marrow transplant recipients: A prospective study. *Clinical and Experimental Immunology*, 72, 267.
- Marchesi, F., Tendas, A., Giannarelli, D., Viggiani, C., Gumenyuk, S., Renzi, D., ... Palombi, F. (2017). Cryotherapy reduces oral mucositis and febrile episodes in myeloma patients treated with high-dose melphalan and autologous stem cell transplant: A prospective, randomized study. *Bone Marrow Transplantation*, 52, 154–156. <https://doi.org/10.1038/bmt.2016.207>
- Nagler, R., Barness-Hadar, L., Lieba, M., & Nagler, A. (2006). Salivary antioxidant capacity in graft versus host disease. *Cancer Investigation*, 24, 269–277. <https://doi.org/10.1080/07357900600634013>
- Navazesh, M., & Kumar, S. K. (2008). Measuring salivary flow: Challenges and opportunities. *The Journal of the American Dental Association*, 139, 35S–40S. <https://doi.org/10.14219/jada.archive.2008.0353>
- Norhagen-Engstrom, G., Hammarstrom, L., Lonnqvist, B., Soder, P. O., & Smith, C. I. (1988). Ontogeny of immunoglobulins in bone marrow-transplanted individuals. An analysis of serum and salivary levels. *Transplantation*, 46, 710–715. <https://doi.org/10.1097/00007890-198811000-00016>
- Peterson, D. E., & Cariello, A. (2004). Mucosal damage: A major risk factor for severe complications after cytotoxic therapy. *Seminars in Oncology*, 31, 35–44. <https://doi.org/10.1053/j.seminoncol.2004.04.006>
- Pink, R., Vondrakova, J., Tvrdy, P., Michl, P., Pazdera, J., Faber, E., ... Indrak, K. (2009). Salivary neutrophils level as an indicator of bone marrow engraftment. *Biomedical Papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia*, 153, 263–269. <https://doi.org/10.5507/bp.2009.051>



- Potting, C., Blijlevens, N., Donnelly, J., Feuth, T., & van Achterberg, T. (2006). A scoring system for the assessment of oral mucositis in daily nursing practice. *European Journal of Cancer Care*, 15, 228–234. <https://doi.org/10.1111/j.1365-2354.2005.00644.x>
- Proctor, G. B. (2016). The physiology of salivary secretion. *Periodontology*, 2000(70), 11–25. <https://doi.org/10.1111/prd.12116>
- Reitamo, S., Konttinen, Y. T., & Segerberg-Konttinen, M. (1980). Distribution of lactoferrin in human salivary glands. *Histochemistry*, 66, 285–291. <https://doi.org/10.1007/BF00495741>
- Slavik, M., Wu, J., & Riley, C. (1993). Salivary excretion of anticancer drugs. *Annals of the New York Academy of Sciences*, 694, 319–321. <https://doi.org/10.1111/j.1749-6632.1993.tb18377.x>
- Voorhees, P. M., & Usmani, S. Z. (2016). The role of high-dose melphalan and autologous stem cell transplant in the rapidly evolving era of modern multiple myeloma therapy. *Clinical Advances in Hematology & Oncology*, 14, 719–728.
- World Medical Association (2001). World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. *Bulletin of the World Health Organization*, 79, 373.

How to cite this article: van Leeuwen SJM, Proctor GB, Potting CMJ, et al. Early salivary changes in multiple myeloma patients undergoing autologous HSCT. *Oral Dis*. 2018;24:972–982. <https://doi.org/10.1111/odi.12866>